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Liquid chromatography–tandem mass spectrometric assay for the quantitation in human plasma of the novel indenoisoquinoline topoisomerase I inhibitors, NSC 743400 and NSC 725776

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ABSTRACT

Topoisomerase I (Topo I) is a recognized target for ovarian, lung, and colorectal cancer therapy. The FDA-approved camptothecin (CPT) Topo I inhibitors, topotecan and irinotecan are labile and their effects are rapidly reversible. The indenoisoquinoline topoisomerase I inhibitors, NSC 743400 and NSC 725776, have been developed as a new generation of Topo I inhibitors and are being advanced to clinical evaluation. To support the clinical development of NSC 743400 and NSC 725776, we developed and validated, according to FDA guidelines, LC–MS/MS assays for the sensitive, accurate and precise quantitation of NSC 743400 and NSC 725776 in 0.2 mL human plasma. After ethyl acetate extraction, separation was achieved with a Synergi Polar RP column and a gradient of 0.1% formic acid in acetonitrile:water. NSC 743400 and NSC 725776 eluted at approximately 3 min, and the total run time was 14 min. Detection consisted of electrospray, positive-mode ionization mass spectrometry. Between 3 and 1000 ng/mL, accuracy was 96.9–108.2% for NSC 743400 and 95.1–106.7% for NSC 725776, and precision was <11.4% for NSC 743400 and <5.9% for NSC 725776. Extraction recovery was >80% for both analytes, and ion suppression ranged from −46.7 to 5.7%. The use of isotopically labeled internal standards and a wash phase at the end of the run were necessary to achieve adequate assay performance. Protein binding in human plasma as assessed by equilibrium dialysis showed both indenoisoquinolines to be more than 98% protein bound.

1. Introduction

Topoisomerase I (Topo I) is a recognized target for ovarian, lung, and colorectal cancer therapy [1]. The FDA-approved camptothecin (CPT) Topo I inhibitors, topotecan and irinotecan (Fig. 1), suffer from lactone instability and rapid reversibility of the Topo I cleavage complexes that they induce [2]. To circumvent these limitations, indenoisoquinoline derivatives have been developed as an alternative class of agents and tested for their ability to inhibit Topo I and tumor growth [2]. NSC 743400 (hydrochloride salt of NSC 724998) and NSC 725776 (Fig. 1), which are two of these novel indenoisoquinoline Topo I inhibitors, are being advanced to clinical evaluation.

In vitro, NSC 743400 and NSC 725776 produced Topo I cleavage at unique genomic positions compared with those resulting from CPT treatment. They cause cell cycle arrest in both S and G(2)-M. As with other known Topo I inhibitors, resistance to NSC 743400 and NSC 725776 was shown in cells deficient for Topo I [2].

The protein-linked DNA breaks characteristic for Topo I poisons were detected in cells treated with nanomolar concentrations of NSC 743400 and NSC 725776. These Topo I cleavage complexes persisted longer after removal of drug (1 μM) when induced by NSC 743400 and NSC 725776 than when induced by CPT or SN-38 (the active metabolite of irinotecan) [2]. In addition to their effects on Topo I, NSC 743400 and NSC 725776 may also exert part of their antitumor effect through antiangiogenesis [3]. Camptothecin, topotecan and SN-38 are good substrates for the efflux pump ABCG2. In contrast, NSC 725776 is only a moderate substrate

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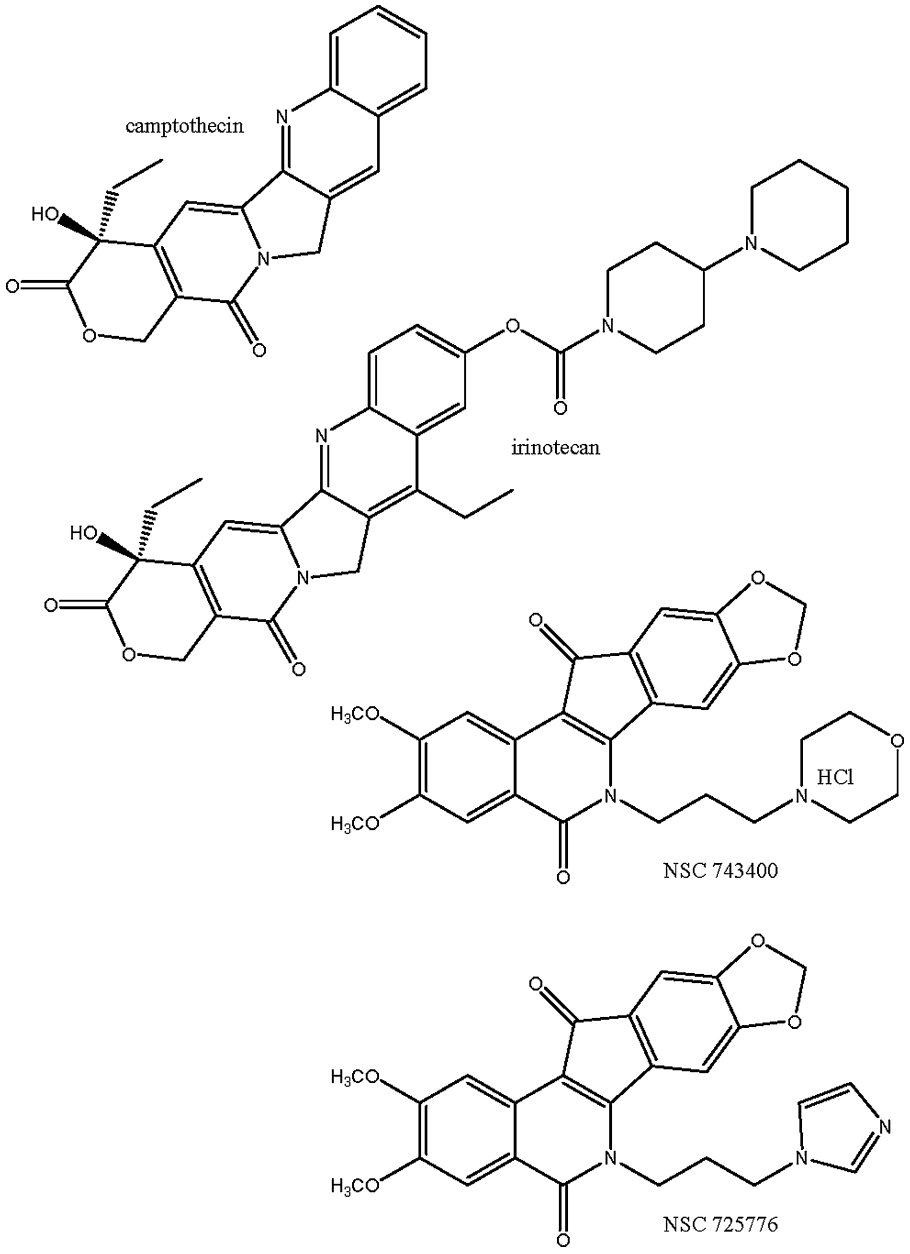

Fig. 1. Structures of camptothecin, irinotecan, NSC 743400, and NSC 725776.

for ABCG2 and ABCB1, and NSC 724998 is not a substrate for either transporter [2]. This suggests that overexpression of these pumps will not likely be a major mechanism of resistance. As a result of their favorable pharmacological characteristics, NSC 743400 and NSC 725776 are promising anticancer drug candidates.

The conventional Topo I inhibitors are sensitive to a pH-dependent reversible conversion between a pharmacologically active lactone form and its inactive, lactone ring-opened, carboxylate form. This equilibrium is also dependent on the protein binding properties of the two forms. Quantitation of these drugs and speciation of both forms is complicated by the chemical instability of the lactone moiety, and requires rapid processing of blood samples and special analytical procedures, as reviewed by Loos et al. [4]. None of these complications apply to the indenoisoquinolines because they do not contain the labile lactone moiety.

To support the clinical development of NSC 743400 and NSC 725776, we developed LC–MS/MS assays to quantitate their concentrations in human plasma. Assay validation was performed for each analyte independently with the same analytical system, under identical conditions, apart from the m/z values monitored.

2. Experimental

2.1. Chemicals and reagents

NSC 743400 ($D_0/D_8 > 99.98\%$), [D_8]-NSC 743400 ($D_8/D_0 > 99.96\%$), NSC 725776 ($D_0/D_3 > 99.97\%$), and [D_3]-NSC 725776 ($D_3/D_0 > 99.98\%$) were provided by the National Cancer Institute (Bethesda, MD, USA). Acetonitrile, methanol and ethyl acetate (all HPLC grade) were purchased from Fisher Scientific (Fairlawn, NJ, USA). Water was purified using a Q-gard® 1 Gradient Milli-Q system (18.2 MΩ cm, Millipore, Billerica, MA, USA). Formic acid was purchased from Sigma–Aldrich (St. Louis, MO, USA). Control human plasma was produced by centrifuging citrate-anticoagulated whole blood (Central Blood Bank, Pittsburgh, PA, USA) for 20 min at $2000 \times g$ at room temperature. Nitrogen for evaporation of sam-

ples was purchased from Valley National Gases, Inc. (Pittsburgh, PA, USA). Nitrogen for mass spectrometrical applications was purified with a Parker Balston Nitrogen Generator (Parker Balston, Haverhill, MA, USA).

2.2. Chromatography

The LC system consisted of an Agilent (Palo Alto, CA, USA) 1100 autosampler and binary pump, a Phenomenex (Torrance, CA, USA) Synergi Polar RP (4 μm, 100 mm × 2 mm) column kept at ambient temperature, and a gradient mobile phase. Mobile phase solvent A was 0.1% (v/v) formic acid in acetonitrile, and mobile phase solvent B was 0.1% (v/v) formic acid in water. The initial mobile phase composition of 50% solvent A and 50% solvent B was maintained for 4 min at a flow rate of 0.2 mL/min. Between 4 and 4.1 min, the percentage of solvent A was increased to 100%, and the flow rate was increased to 0.4 mL/min. Between 4.1 and 8 min, the percentage of solvent A was maintained at 100%. Between 8 and 8.1 min, the percentage of solvent A was decreased to 50%, and the flow rate was increased to 0.5 mL/min. These conditions were maintained until 14 min, followed by injection of the next sample. Total run time was 14 min.

2.3. Mass spectrometry

Mass spectrometric detection was carried out using a Waters (Milford, MA, USA) Quattromicro triple-stage, benchtop quadrupole mass spectrometer with electrospray ionization in positive-ion, multiple reaction monitoring (MRM) mode. The settings of the mass spectrometer were as follows: capillary voltage 4.0 kV; cone voltage 30.0 V; source temperature 120 °C; and desolvation temperature 350 °C. The cone and desolvation gas flows were 100 and 550 L/h, respectively. The collision voltage was 25 V. Quadrupoles 1 and 3 each had low mass and high mass resolution set at 12.0. The dwell time was 0.25 s, and the interscan delay was 0.2 s. The span was set at 0 a.m.u. The MRM *m*/*z* transitions monitored were: 479.4–392.0 for NSC 743400; 487.4–392.0 for [D_8]-NSC 743400; 460.0–392.0 for NSC 725776; and 463.0–392.0 for [D_3]-NSC 725776 (see Fig. 2 for proposed fragmentation). The LC system and mass spectrometer were controlled by Waters MassLynx software (version 4.0), and data were collected with the same software.

2.4. Preparation of calibration standards and quality control samples

Stock solutions of NSC 743400 and NSC 725776 and their respective deuterated internal standards were prepared independently at 1 mg/mL in 0.1% (v/v) formic acid in water and stored at −80 °C. On assay days, these solutions were serially diluted (in steps of 10-fold) with 0.1% (v/v) formic acid in water to obtain the lower calibration working solutions. These calibration working solutions were diluted in human plasma to produce the following concentrations of either NSC 743400 or NSC 725776: 3, 10, 30, 100, 300, 500, 750, and 1000 ng/mL. For each calibration series, zero and blank samples were also prepared from 200 μL of control plasma.

Quality control (QC) stock solutions were prepared independently from separate weighings of NSC 743400 and NSC 725776 and stored at −80 °C. These solutions were diluted in human plasma to produce the following QC samples of either NSC 743400 or NSC 725776: QC low (QCL) 5 ng/mL; QC mid (QCM) 200 ng/mL; and QC high (QCH) 800 ng/mL.

2.5. Sample preparation

Ten microliters of 10 μg/mL of the respective deuterated internal standard in 0.1% (v/v) formic acid in water and 1 mL of ethyl acetate were added sequentially to each tube of 200 μL standard, QC, or sample plasma. Samples were vortexed for 1 min on a Vortex Genie-2 set at 10 (Model G-560 Scientific Industries, Bohemia, NY, USA) and then centrifuged at 14,000 × *g* at room temperature for 5 min. The resulting supernatants were transferred to 12 mm × 75 mm borosilicate glass tubes and evaporated to dryness under a stream of nitrogen at 37 °C. Dried residues were redissolved in 100 μL of acetonitrile: water: formic acid (50:50:0.1, v/v/v). The solutions were transferred to microcentrifuge tubes and centrifuged for 3 min at 14,000 × *g*. The supernatants were transferred to autosampler vials, followed by injection of 3 μL into the LC–MS/MS system.

2.6. Validation procedures

2.6.1. Calibration curve and lower limit of quantitation (LLQ)

Decreasing concentrations of NSC 743400 and NSC 725776 were injected into the analytical system to determine the minimal concentration with a signal-to-noise ratio of at least 5:1, and adequate precision and accuracy. Calibration standards and blanks were prepared (see Section 2.4) and analyzed in triplicate to establish the calibration range with acceptable accuracy and precision. The analyte-to-internal standard ratio (response) was calculated for each sample by dividing the area of the analyte peak by the area of the internal standard peak. Standard curves of NSC 743400 and NSC 725776 were constructed individually by plotting the analyte-to-internal standard ratio *versus* the known concentration of NSC 743400 or NSC 725776, respectively, in each sample. Standard curves were fit by linear regression with weighting by $1/y^2$, followed by back-calculation of concentrations. The deviations of these back-calculated concentrations from the nominal concentrations, expressed as percentage of the nominal concentration, reflected the performance of the calibration curve.

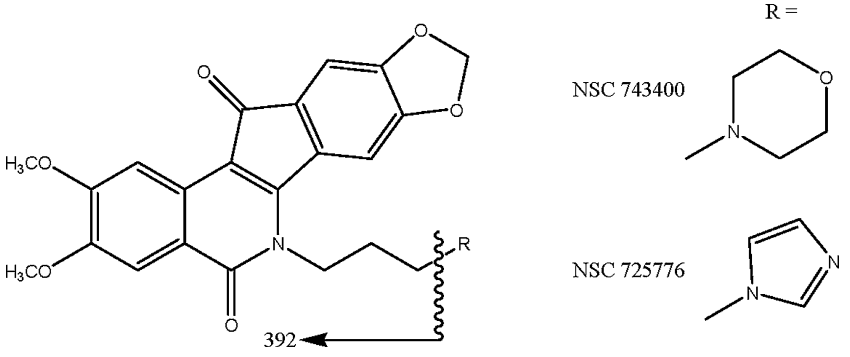

Fig. 2. Common fragmentation product ion of NSC 743400 ([M+H]$^+$ *m*/*z* 479.4–392.0) and NSC 725776 ([M+H]$^+$ *m*/*z* 460.0–392.0).

2.6.2. Accuracy and precision

The accuracy and precision of the assay were determined by analyzing samples with NSC 743400 or NSC 725776 at the LLQ, QCL, QCM, and QCH concentrations in 6 replicates each in 3 analytical runs, together with independently prepared, triplicate calibration curves. Accuracy was calculated at each test concentration as:

$$\frac{\text{mean measured concentration}}{\text{nominal concentration}} \times 100\%.$$

Assay precision was calculated by ANOVA as described [5], by using SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA). Back-calculated concentrations of calibration and QC samples were entered with the run number as factor. From the resulting mean squares of the within runs and mean squares of the between runs, the intra-assay and inter-assay precisions were calculated.

2.6.3. Selectivity and specificity

To investigate whether endogenous matrix constituents interfered with the assay, six individual batches of control, drug-free human plasma were processed and analyzed according to the described procedures. Responses of NSC 743400 and NSC 725776 at the LLQ concentrations were compared with the response of the blank samples. Although NSC 743400 and NSC 725776 are analyzed separately, we did characterize the cross-talk of QCH samples of each analyte when analyzed in the assay of the other analyte.

2.6.4. Extraction recovery and ion suppression

We determined the extraction recoveries of NSC 743400 and NSC 725776 from plasma by comparing the absolute response of an extract of control plasma to which these analytes had been added after extraction, with the absolute response of an extract of plasma to which the same amounts had been added before extraction. The ion suppression of NSC 743400 and NSC 725776 by plasma matrix components was defined as the decrease of the absolute response of an extract of control plasma to which NSC 743400 or NSC 725776 had been added after the extraction relative to the absolute response of reconstitution solvent to which the same amount of each respective analyte had been added. Experiments were performed at the three QC concentrations, in triplicate.

2.6.5. Stability

Long-term stability experiments were performed in plasma and in stock solution after storage at −80 °C. Stability in the stock solution was expressed as the percentage recovery of the stored solution relative to a fresh solution. The stabilities of NSC 743400 and NSC 725776 in plasma at −80 °C were determined by assaying samples before and after storage. In addition, the stabilities of NSC 743400 and NSC 725776 in stock solution at room temperature for 4 h were determined in triplicate. All stability testing in plasma was performed in triplicate at the QCL, QCM and QCH concentrations. The effect of 3 freeze/thaw cycles on NSC 743400 and NSC 725776 concentrations in plasma was evaluated by assaying samples after they had been frozen (−80 °C) and thawed on 3 separate days and comparing the results with those of freshly prepared samples. The stabilities of NSC 743400 and NSC 725776 in plasma during sample preparation were evaluated by assaying samples before and after 4 h of storage at room temperature. To evaluate the stabilities of NSC 743400 and NSC 725776 in reconstituted samples in the autosampler, we re-injected QC samples and calibration curves approximately 72 h after the first injection and compared the concentrations derived from the second injection with those derived from the first injection. The results of the second runs were expressed as a percentage of their respective values in the first runs.

2.6.6. Parallelism

To demonstrate parallelism, the ability to dilute samples from above the upper limit of quantitation to within the validated concentration range, plasma samples containing NSC 743400 or NSC 725776 above the upper limit of quantitation were diluted to within the assay range. Plasma samples ($N = 3$) with NSC 743400 and NSC 725776 concentrations of 5 and 10 μg/mL were diluted 10- and 20-fold with control plasma and assayed.

2.7. Application of the assay

To show the applicability of the assay, we assessed the protein binding at 4000 and 10,000 ng/mL of NSC 743400 or NSC 725776 in human plasma, and tissue culture medium with 10% fetal bovine serum. Protein binding was assessed by rapid equilibrium dialysis against PBS in Pierce RED devices (Thermo Fisher Scientific, Rockford, IL, USA) with an 8000 Da molecular weight cut-off. RED devices were incubated for 24 h at 37 °C in replicates of 4. Samples were diluted in control human plasma and analyzed as detailed above.

3. Results and discussion

3.1. Method development

Previously, we developed an LC–MS/MS assay to quantitate NSC 724998 (the base of NSC 743400) in dog plasma. This assay used NSC 725776 as the internal standard [6]. Upon applying this method to human plasma, the assay precision was unacceptable, even after we started using stable-labeled isotopic analogues as internal standard. Changing the gradient system to effect later elution did not improve assay performance. Substitution of the Synergi Hydro-RP 80 A column used in the assay for dog plasma for a Synergi Polar RP column resulted in better retention and adequate performance of the assay, even when operated under isocratic conditions. Because of the observed matrix effects (the plasma matrix enhances the ionization of each indenoisoquinoline), we included a column wash at the end of each run.

3.2. Validation of the assay

3.2.1. Chromatography

NSC 743400 and NSC 725776 had retention times of approximately 2.8 and 2.9 min, respectively, corresponding to capacity factors of 3.3 and 3.5, respectively (with a void time of 0.65 min). The respective deuterated internal standards eluted at retentions identical to their natural isotope analogues. Representative chromatograms of NSC 743400 and NSC 725776 (at the LLQ), and internal standards in plasma are displayed in Fig. 3.

3.2.2. Calibration curve and LLQ

According to the FDA guidelines for bioanalytical method validation [7], the calibration curve describes the concentration *versus* response relationship adequately if the observed deviation and precision are ≤20% for the LLQ and ≤15% for all other calibration concentrations. At least 4 of 6 calibration points should meet the above criteria [7].

The selected assay range of 3–1000 ng/mL fulfilled the FDA criteria for the LLQ concentration and the calibration curve. Accuracies and precisions at the different calibration concentrations were determined from triplicate calibration curves on 3 separate days and are reported in Table 1. At most concentrations, the mean square of the within runs was greater than the mean square of the between runs, indicating that there was no significant additional variability due to the performance of the assay in different runs [5].

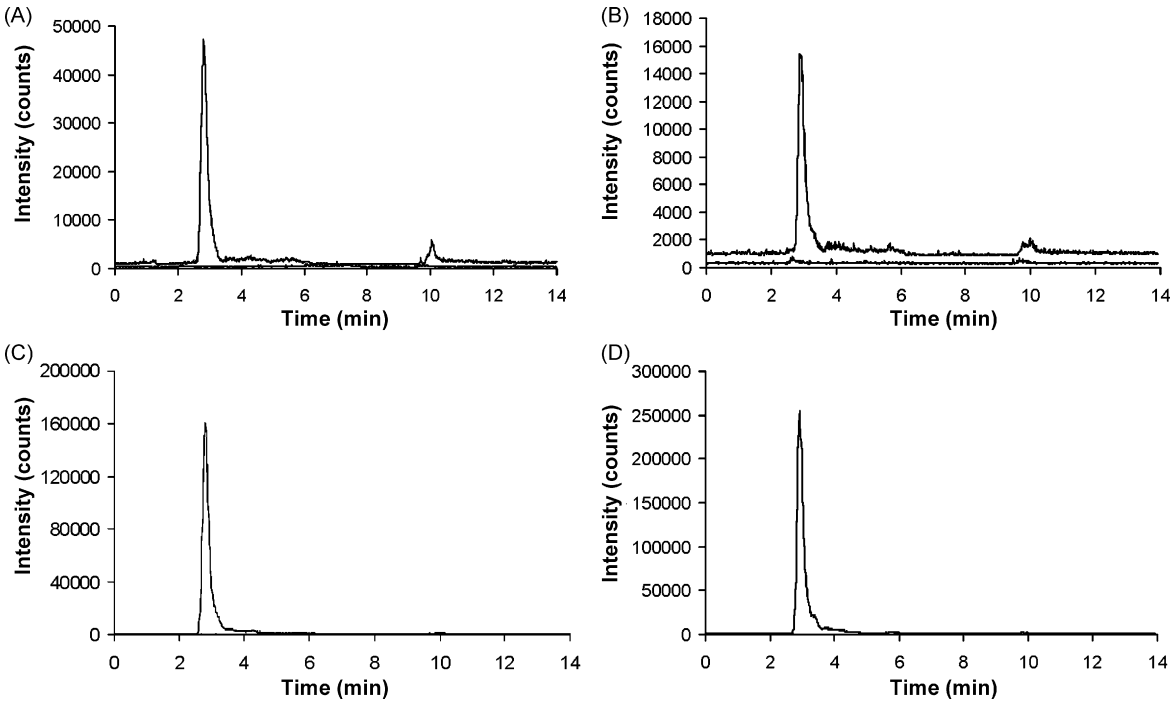

Fig. 3. Representative chromatograms of: (A) NSC 743400 (m/z 479.4–392.0; 2.8 min) added to control plasma at the LLQ concentration of 3 ng/mL (top trace with an offset of 500 counts) and control human plasma (bottom trace); (B) NSC 725776 (m/z 460.0–392.0; 2.9 min) added to control plasma at the LLQ concentration of 3 ng/mL (top trace with an offset of 500 counts) and control human plasma (bottom trace); (C) [D_8]-NSC 743400 internal standard (m/z 487.4–392.0; 2.8 min) added to control plasma at the a concentration of 500 ng/mL; D) [D_3]-NSC 725776 internal standard (m/z 463.0–392.0; 2.9 min) added to control plasma at a concentration of 500 ng/mL.

Representative calibration curves and corresponding correlation and regression coefficients are shown in Fig. 4.

3.2.3. Accuracy and precision

FDA guidelines specify that the accuracies for all tested concentrations should be within ±15%, and the precisions should not be >15% CV except for the LLQ, in which case these parameters should not exceed 20% [7].

The accuracies and intra- and inter-assay precisions for the tested concentrations (LLQ, QCL, QCM, QCH) were all within the defined acceptance criteria (Table 2).

Table 1
Assay performance data of the calibration samples for NSC 743400 and NSC 725776 in human plasma.

Analyte	Concentration (ng/mL)	Accuracy (%)	Intra-assay precision (%)	Inter-assay precision (%)
NSC 743400	3	102.4	9.3	–[a]
	10	97.2	8.5	–[a]
	30	101.2	10.6	–[a]
	100	102.6	8.5	–[a]
	300	104.3	8.9	3.2
	500	99.9	12.1	–[a]
	750	99.9	8.0	–[a]
	1000	102.4	9.2	3.5
NSC 725776	3	103.4	9.8	–[a]
	10	95.6	8.3	–[a]
	30	97.9	6.8	3.4
	100	99.5	6.1	–[a]
	300	101.3	9.3	–[a]
	500	103.5	5.6	2.5
	750	102.4	7.5	–[a]
	1000	105.4	3.3	2.8

$N = 9$; triplicate results, each in 3 separate runs, for each concentration.

[a] The mean square of the within runs was greater than the mean square of the between runs, indicating that there was no significant additional variation due to the performance of the assay in different runs [5].

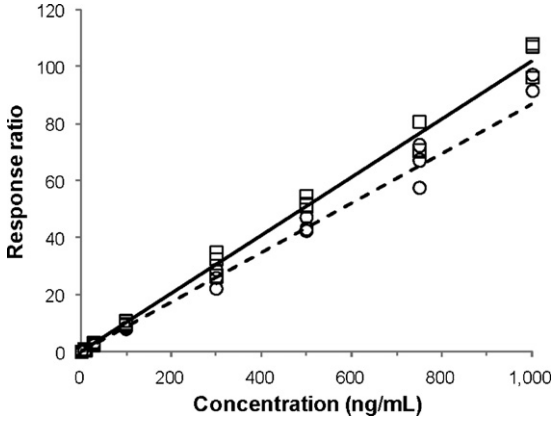

Fig. 4. Representative calibration curves ($N = 3$ for each concentration) used to quantitate NSC 743400 (□, solid line), and NSC 725776 (○, dashed line) in human plasma samples (response NSC 743400 = 0.102·conc. + 0.0119; $R^2 = 0.9932$. response NSC 725776 = 0.0870·conc. + 0.0065; $R^2 = 0.9923$).

Table 2
Assay performance data for the quantitation of LLQ, QCL, QCM and QCH NSC 743400 and NSC 725776 concentrations in human plasma.

	Concentration (ng/mL)	Accuracy (%)	Intra-assay precision (%)	Inter-assay precision (%)
NSC 743400	3 (LLQ)	96.9	10.2	4.1
	5 (QCL)	102.4	7.3	3.8
	200 (QCM)	108.2	3.5	1.3
	800 (QCH)	101.5	11.4	6.1
NSC 725776	3 (LLQ)	106.7	5.8	3.2
	5 (QCL)	106.0	5.1	–[a]
	200 (QCM)	100.9	8.1	–[a]
	800 (QCH)	95.1	5.9	0.6

$N = 18$; 6-fold results, each in 3 separate runs, for each concentration.

[a] The mean square of the within runs was greater than the mean square of the between runs, indicating that there was no significant additional variation due to the performance of the assay in different runs [5].

Table 3
Recoveries of NSC 743400 and NSC 725776 from human plasma and their respective ion suppressions in human plasma extract, with coefficients of variation (CV).

	Concentration (ng/mL)	Recovery (%)	CV (%)	Ion suppression (%)	CV (%)
NSC 743400	5 (QCL)	89.0	24.7	−40.4	17.9
	200 (QCM)	80.1	10.4	−46.7	8.5
	800 (QCH)	123.4	15.9	−6.2	16.4
NSC 725776	5 (QCL)	112.2	14.9	−12.2	13.6
	200 (QCM)	92.6	19.6	5.7	22.9
	800 (QCH)	80.4	22.0	−31.8	10.5

$N = 3$, for each concentration.

Table 4
Stability of NSC 743400 and NSC 725776 under varying conditions.

Storage condition	Concentration (ng/mL)		Stability (%)	CV (%)	Replicates
NSC 743400					
Stock solution 4 h					
Ambient temp.		1,000,000	104.7	3.3	3
Stock solution 5 months					
−80 °C		1,000,000	112.4	13.8	3
Plasma 4 h					
Ambient temp.	QCL	5	97.3	4.3	3
	QCM	200	105.4	4.4	3
	QCH	800	99.3	3.1	3
Plasma 3 freeze–thaw cycles					
−80 °C	QCL	5	102.4	5.3	3
	QCM	200	97.8	4.3	3
	QCH	800	100.8	2.9	3
Plasma 7 months					
−80 °C	QCL	5	105.2	4.4	4
	QCM	200	99.7	3.1	4
	QCH	800	99.8	8.4	4
NSC 725776					
Stock solution 4 h					
Ambient temp.		1,000,000	102.0	5.2	3
Stock solution 7 months					
−80 °C		1,000,000	100.3	6.6	3
Plasma 4 h					
Ambient temp.	QCL	5	94.3	6.9	3
	QCM	200	98.9	4.4	3
	QCH	800	100.4	2.9	3
Plasma 3 freeze–thaw cycles					
−80 °C	QCL	5	104.6	4.0	3
	QCM	200	99.9	4.8	3
	QCH	800	98.4	4.8	3
Plasma 7 months					
−80 °C	QCL	5	94.5	5.4	4
	QCM	200	98.5	6.9	4
	QCH	800	91.2	3.1	4

3.2.4. Selectivity and specificity

According to FDA guidelines, the signal at the LLQ must be at least 5 times the signal of any co-eluting peaks [7].

Chromatograms of six individual control plasma samples contained no co-eluting peaks >20% of the analyte areas at the LLQ concentration (interference <12.8% for NSC 743400 and <18.2% for NSC 725776) (Fig. 3). In subsequent analyses, there were no interfering or co-eluting peaks. NSC 725776 generated <0.01% cross-talk into the NSC 743400 MRM channel. NSC 743400 generated <0.07% cross-talk into the NSC 725776 MRM channel.

3.2.5. Extraction recovery and ion suppression

FDA-guidelines require that recovery be consistent and precise [7]. A recovery of ≥70% with a variation of 15% is generally accepted [5,7]. There is no specific guideline for the percentage of ion suppression that is acceptable. Ultimately, the assay performance, as expressed in the precision and accuracy, is most relevant; however, a large and/or variable ion suppression may explain an unsatisfactory assay performance.

The recoveries of both NSC 743400 and NSC 725776 were >80%, with CVs between 10.4 and 24.7%. Ion suppression ranged from −6.2 to −46.7% (i.e. ionization enhancement), with CVs between 8.5 and 17.9% (NSC 743400), and from −31.8 to 5.7%, with CVs between 10.5 and 22.9% (NSC 725776) (Table 3).

3.2.6. Stability

Stability in biological samples is acceptable when ≥85% of the analyte is recovered.

The stabilities of the NSC 743400 and NSC 725776 stock solutions at room temperature for 4 h were 104.7 and 102.0%, respectively (Table 4). Stabilities in stock solutions for >5 months at −80 °C were 112.4 and 100.3% for NSC 743400 and NSC 725776, respectively. The stabilities of NSC 743400 and NSC 725776 in plasma during freeze–thaw cycling and in plasma at room temperature (>94.3% after 4 h) were also acceptable. Long-term stabilities of NSC 743400 and NSC 725776 in plasma at −80 °C were adequate with recoveries between 91.2 and 105.2%. The absolute responses of plasma extracts of NSC 743400 at the calibration concentrations, when reconstituted and kept in the autosampler for 72 h, were 118.8 to 140.4% of the initial responses (CV 16.5–25.0%), while the response of NSC 743400 relative to the internal standard signal ranged from 93.1 to 98.5% (CV 5.8–8.1%). The absolute responses of plasma extracts of NSC 725776 at the calibration concentrations, when reconstituted and kept in the autosampler for 72 h, were 149.6–151.0% of the initial responses (CV 10.0–11.1%), while the response of NSC 725776 relative to the internal standard signal ranged from 98.7 to 103.5% (CV 8.2–8.9%).

3.2.7. Parallelism

The mean accuracies of the diluted samples of NSC 743400 were 98.6%, with a CV of 1.4% at 5000 ng/mL, and 101.3%, with a CV

Table 5
Protein binding of NSC 743400 and NSC 725776 in human plasma, dog plasma and tissue culture medium with 10% fetal bovine serum ($N = 4$).

	Concentration NSC 743400 (ng/mL)			
	% Free4000	% Free10,000	% Bound4000	% Bound10,000
Human plasma (%CV)	0.4 (31)	0.4 (28)	99.6 (0.1)	99.6 (0.1)
Tissue culture medium (%CV)	6.8 (39)	1.5 (18)	93.6 (2.8)	98.5 (0.3)
	Concentration NSC 725776 (ng/mL)			
	% Free4000	% Free10,000	% Bound4000	% Bound10,000
Human plasma (%CV)	1.3 (78)	0.59 (70)	98.7 (1.0)	99.4 (0.4)
Tissue culture medium (%CV)	0.53 (26)	0.20 (28)	99.5 (0.1)	99.8 (0.1)

of 1.0% at 10,000 ng/mL. The mean accuracies of the diluted samples of NSC 725776 were 102.9%, with a CV of 4.3% at 5000 ng/mL, and 100.7%, with a CV of 1.3% at 10,000 ng/mL. These results indicate parallelism for both the NSC 743400 and NSC 725776 assay.

3.3. Application of the assay

Results for the protein binding are shown in Table 5. Protein binding in human plasma was high, being >98% for both NSC 743400 and NSC 725776.

4. Conclusion

Our objective was to develop and validate an analytical method for the quantitation of NSC 743400 and NSC 725776 in human plasma. We accomplished this using reversed phase chromatography equipped with triple quadrupole mass spectrometric detection.

The method presented here allows the quantitation of NSC 743400 and NSC 725776 in human plasma and, to our knowledge, is the first assay for NSC 743400 and NSC 725776 published to date, that is validated according to FDA guidelines [7].

The application of our study to protein binding leads to some interesting conclusions. NSC 743400 displayed 72 h IC_{50} values of 0.3, 0.56, and 1.2 μM against P388, MCF-7, and HCT-116 cells, while NSC 725776 displayed 72 h IC_{50} values of <0.03, 0.09, and 0.13 μM, respectively [2]. However, the IC_{50} values as determined *in vitro* may overestimate the potency of these compounds when applied *in vivo*. Because the free fractions of NSC 743400 and NSC 725776 in tissue culture medium are much higher than those in human plasma, the target plasma concentrations to be achieved in clinical trials may well be much higher than expected from simple extrapolation of *in vitro* data. In addition, the reported IC_{50} values and our assessment of protein binding in tissue culture medium imply that NSC 725776 is even more potent than suggested by merely the IC_{50} values.

The analytical method presented in this paper will be a valuable tool in quantitating NSC 743400 and NSC 725776 plasma concentrations as they each undergo full clinical development.

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